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Early transient presence of implanted bone marrow stem cells reduces lesion size after cerebral ischaemia in adult rats

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Aims: Previous studies on the therapeutic time window for intravascular administration of bone marrow stem cells (BMSCs) after stroke have shown that early intervention (from 3 h after onset) in the middle cerebral artery occlusion (MCAO) rat model is the most effective approach to reduce ischaemic lesion size. We have confirmed these observations but noticed that 2 weeks after transplantation, almost none of the grafted BMSCs could be detected in or around the lesion. The present experiments aimed to assess the fate and kinetics of intravascularly injected BMSCs shortly after administration in correlation to the development of the ischaemic lesion after MCAO. **Methods:** We administered a syngeneic suspension of complete (haematopoietic and mesenchymal) BMSCs via the carotid artery to rats at 2 h after MCAO onset. We examined the distribution and tissue location of BMSCs

within the first 24 h after arterial administration by perfusion-fixating rats and performing immunohistochemical analysis at different time points. **Results:** The vast majority (>95%) of BMSCs appeared to become trapped in the spleen shortly after injection. Six hours after implantation, together with the appearance of activated microglia, the first BMSCs could be detected in and around the lesion; their number gradually increased during the first 12 h after implantation but started to decrease at 24 h. The implanted BMSCs were surrounded by activated and phagocytotic microglia. **Conclusion:** Our results show that ischaemic lesion size reduction can already be achieved by the early transient presence at the lesion site of intravascularly implanted BMSCs, possibly mediated via activated microglia.

Keywords: mesenchymal stem cells, microglia, middle cerebral artery occlusion, neuroregeneration, penumbra, stroke

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Introduction

Ischaemic stroke is one of the neurological disorders that has been examined in experimental animals as a potential

target for stem cell therapy to enhance long-term stroke recovery as a more promising alternative to largely unsuccessful drug approaches [1,2]. In view of the claimed pluripotency of both haematopoietic and mesenchymal bone marrow stem cells (BMSCs), their ability to cross the blood–brain barrier and their relatively easy autologous accessibility, these cells have been considered as prominent candidate stem cells. Intracerebral, intravenous or

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intra-arterial administration of BMSCs starting from 24 h after transient middle cerebral artery occlusion (MCAO) has been demonstrated to improve the functional outcome in this experimental ischaemic stroke model (for reviews [3–7]). Even delayed treatment at 7 days or even at 1 month after stroke onset was reported to increase brain plasticity and to improve long-term functional outcome. The mechanism of the neurorestorative effect of BMSCs initially has been merely ascribed to their claimed pluripotency and their ability to differentiate into brain cell lineages, and so replacing lost astrocytes, oligodendrocytes, neurons and endothelial cells. However, the number of implanted BMSCs and their derivatives recovered at the lesion site appeared to be highly variable, and was in most cases not correlated to the extent of the beneficial effects. Instead, growing evidence suggests that the secretory products of BMSCs may be the active agents. BMSCs secrete a wide variety of growth factors (e.g. VEGF, bFGF and BDNF) that support angiogenesis, neurogenesis and synaptic plasticity, and by doing so promote the functional outcome [8–11]. In general, however, despite an improvement in functional readout parameters, the core of the ischaemic lesion appeared hardly reduced in size after these non-acute injections of BMSCs; an increased rescue and protection of neurons in the penumbra region and an increased synaptic plasticity seemed to account for the functional improvement observed after stroke.

A study on the therapeutic time window for acute intra-vascular administration of BMSCs by Iihoshi *et al.* [12], however, showed that very early intervention in the MCAO model in the rat (that is, ranging from 3 to 12 h after stroke onset) indeed can reduce the actual lesion size. Infusion of BMSCs as early as 3 h after a MCAO of 45 min almost completely abolished the lesion with only some inflammatory responses visible [12]. Two weeks after transplantation, Iihoshi *et al.* found that BMSCs had accumulated in and around the site of the ischaemic lesion, with some of them expressing neuronal and astrocytic markers [12], although the actual percentages and the significance level remained unclear. The optimal 3 h time frame described in their experimental study may be of clinical interest, as it coincides with the time window given to stroke patients for acute successful intra-arterial thrombolysis [13]. The present experiment was designed to assess the fate and kinetics of intravascularly injected BMSCs shortly after administration in correlation to the development of the ischaemic lesion after MCAO, including neuronal degeneration and microglia response.

Materials and methods

Animals

In total, 65 male inbred Wistar rats (Charles-River Laboratories, Wilmington, MA, USA) with a bodyweight ranging between 350–400 g were used. The rats had free access to water and food before and after the procedures. All animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and with regulations of the local Experimental Animal Committee.

BMSC preparation

For the preparation of BMSC suspensions, a littermate of the recipient rat was quickly terminated by cervical dislocation under halothane anaesthesia. The femur was rapidly dissected and completely cleaned from adherent tissue. After removing the knee joint, a needle connected to a 1 ml syringe, with 1 ml of basic alpha medium (Invitrogen, Breda, The Netherlands) plus 5% FCS (foetal calf serum), was inserted through the knee plane into the femur. Both the needle and the femur were placed in a 5 ml tube and the medium was firmly injected into the femur and vigorously flushed two times. Subsequently, the cell suspension was mechanically disassociated by repeatedly flushing the suspension through the needle. The cells were then placed on ice and erythrocytes were lysed with erythrocyte lysis buffer (8.26 g NH_4Cl , 1 g NH_4HCO_3 , 0.0037 g EDTA in 1 l of aqua dest), 3 ml per 50 million cells, during 10 min. Cells were washed three times in PBS + 0.2% BSA and in PBS, counted and re-suspended ($10^7/\text{ml}$) in basic alpha medium plus 5% FCS. Cells were incubated overnight at 37°C in this medium in order to remove T cells and monocytes and to label the cells with a rhodamine-dextran conjugate ('mini-Ruby'; 20 $\mu\text{g}/\text{ml}$). Next day, the cells were washed and counted for a final concentration of 10^7 cells/100 μl in saline. This procedure yielded a pure CD34/CD45-positive mononuclear BMSC fraction, with a rhodamine-dextran labelling efficiency of over 98%.

Focal cerebral ischaemia Rats were anaesthetized with a nitrous oxide/oxygen/halothane mixture (69%/30%/1%) administered through an inhalation mask. During the whole procedure, rats were kept on a temperature of 37°C by placing them on a heating pad. MCAO was induced by

the intraluminal filament technique [14]. In brief, the left carotid bifurcation was exposed, and the external carotid artery (ECA) was coagulated distal to the bifurcation. Close to the bifurcation, a ligature was loosely tied around the ECA. A 40-mm 3-0 nylon monofilament (Rico, Debrecen, Hungary) with a heat-blunted spherical tip was inserted through a small opening in the stump of the ECA and gently advanced (approximately 20 mm) through the internal carotid artery (ICA) until it blocked the middle cerebral artery. After 1 h of occlusion, the intraluminal monofilament was gently withdrawn, the ligature around the ECA was tightened, reperfusion was restored and the wound was closed temporarily to enable BMSC administration via the ECA after 1 h of reperfusion.

Intra-arterial administration of BMSCs After 1 h, the wound was re-opened, clips were placed on the ICA and the common carotid artery and, along the ligature, a blunt needle connected to a catheter was inserted into the ECA. The catheter was filled with saline to prevent air bubbles and was connected to a syringe filled with labelled stem cells. The ligature around the ECA was tightened around the blunt needle and the clip on the ICA was removed. The connection was tested by gently retrieving some blood from the ECA. Then, 100 µl of saline containing 1 000 000 cells (in control groups just saline) was slowly injected through the blunt needle. After injection, the clip was placed back onto the ICA and the blunt needle was extracted from the ECA. The ECA was coagulated below the opening to prevent bleeding. The clips were removed to reperfuse the brain and the incision was cleaned and closed.

Perfusion fixation At various time points after MCAO and BMSC administration, rats were anaesthetized with pentobarbital and intracardially perfused with approximately 250–300 ml of 4% paraformaldehyde in 0.1 M PBS (pH = 7.4). The complete brain, including cerebellum, was excised and post-fixed in 4% paraformaldehyde for 48 h, then changed to 0.1 M PBS containing 0.1% azide. Samples from liver, lung and spleen were also collected in order to analyse the presence of transplanted BM stem cells in these organs.

Immunohistochemistry For immunohistochemical analysis of the BMSC-treated stroke-induced brain lesions, 14-µm-thick coronal sections were cut on a cryostat microtome (Leica, Rijswijk, the Netherlands). Sections were collected on gelatine/poly-L-lysine-coated glass slides

starting from Bregma –1.6 mm [15], that is, at the level that the left–right connection of the corpus callosum was visible, throughout the striatum up to Bregma 2.4 mm. Random 14-µm-thick sections were cut from the liver, spleen and lung specimens.

In order to optimize the exposure of, in particular, nuclear antigens to the primary and secondary antibodies during the immunohistochemical staining procedure, a number of sections were subjected to an antigen retrieval method. Glass slides with sections were placed in a cuvet containing 10 mM sodium citrate and heated in a microwave for 5 min on 20% capacity. After cooling the cuvet at room temperature for 5 min, the sodium citrate was removed and replaced by ice-cold 0.1 M PBS for 5 min.

Besides a histochemical overview staining with cresyl-violet, we have analysed the size and the cellular composition of the ischaemic lesion in the striatum and the consequences of BM stem cell treatment with various primary antibodies in immunohistochemical procedures. We identified dying neurons in the core of the lesion with anti-activated caspase-3 and still viable neurons in the penumbra with anti-NeuN. The penumbra contains neurons that have been injured but might still have the capacity for regeneration; re-outgrowth of their neurites was detected using anti-GAP43, a growth-associated protein. Degenerating cells in the lesion recruit/attract microglia, which we identified by using anti-Iba-1 (ionized calcium binding adapter 1, Wako Chemicals GmbH, Neuss, Germany). The implanted BMSCs were labelled with a red fluorescent rhodamine–dextran conjugate (mini-Ruby, see above), which enables their detection in the brain sections. Additionally, we have used anti-CD34 and anti-CD45 to confirm the identity of the red-fluorescent, grafted BM stem cells. In order to detect grafted BMSCs that might have differentiated into a neural cell type, we have examined the co-labelling of mini-Ruby with immunostaining after incubation with anti-MAP2 (neuron), anti-GFAP (astrocyte) and anti-RIP (oligodendrocyte). Although most immunoreactivity was detected with fluorescence using proper, fluorescently labelled secondary antibodies, in some instances peroxidase-mediated diaminobenzidine staining was performed. In these cases, endogenous peroxidase was blocked in a pre-treatment with 0.3% H₂O₂ in PBS during 30 min.

(Immuno-)histometrical analysis Immunofluorescent sections were analysed under a Leica (SP2 AOBS) Confocal Laser Scan Microscope and a Zeiss (Axioscope 2)

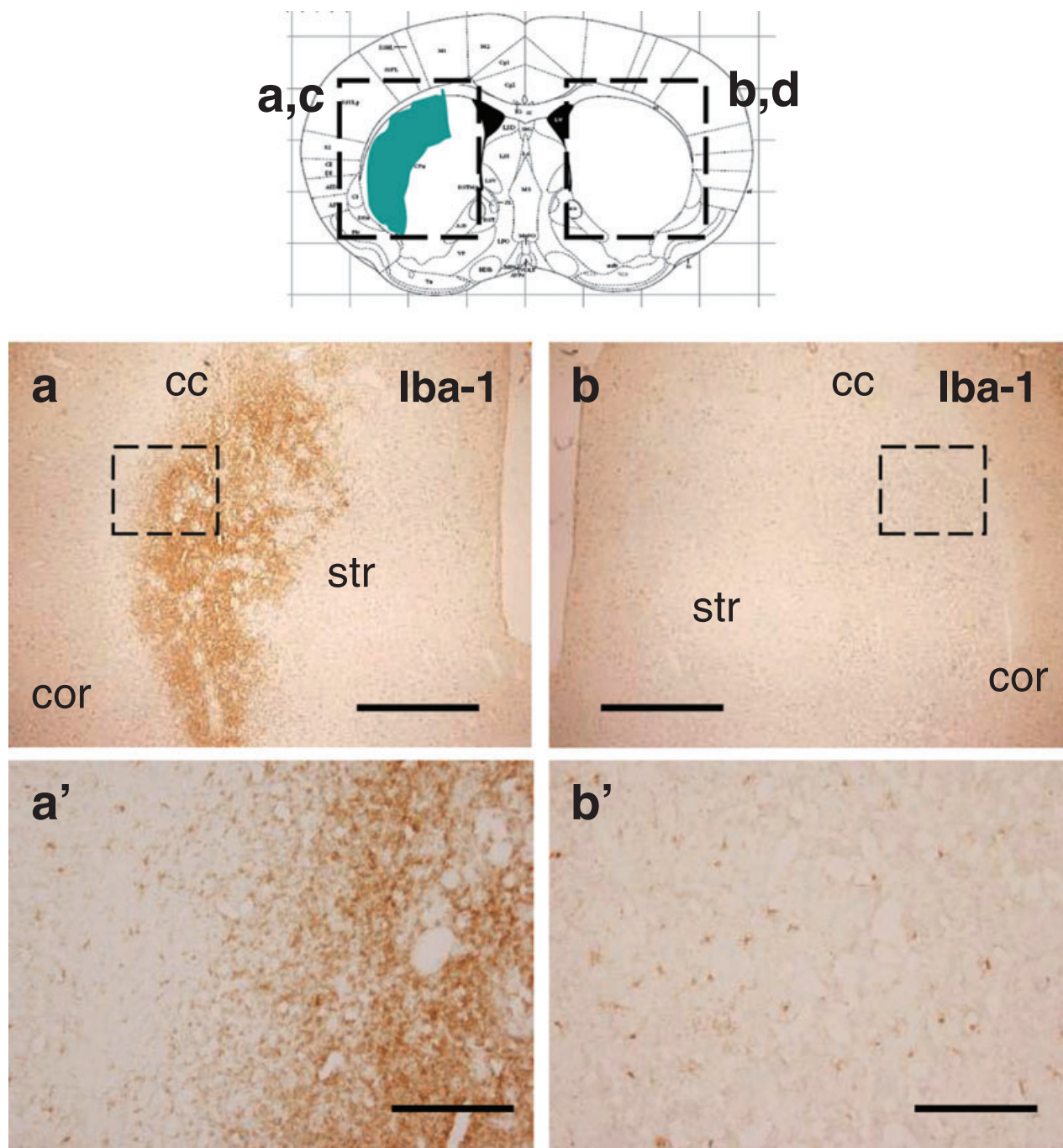


Figure 1. Ischaemic lesion in striatum 2 weeks after middle cerebral artery occlusion. A schematic drawing of brain section shows the striatal areas depicted in **a–d**. (**a,a'**) Iba-1 immunostaining (brown) for activated microglia clearly delineates the lesion in the left side of the striatum; magnification of indicated area in **a'**. (**b,b'**) In contrast, the contralateral unaffected side of the striatum only shows a homogenous distribution of quiescent microglia; magnification of indicated area in **b'**. (**c,c'**) Immunostaining with anti-NeuN (brown), which labels nuclei of viable neurons, shows the degeneration of neurons in the ischaemic striatal lesion; magnification of indicated area in **c'**. (**d,d'**) In the contralateral striatum, NeuN-labelled neurons lay distributed throughout the unaffected striatum; magnification of indicated area in **d'** (cor, cortex; cc, corpus callosum; str, striatum). Bars in **a–d**, 1 mm; in **a'–d'**, 0.1 mm.

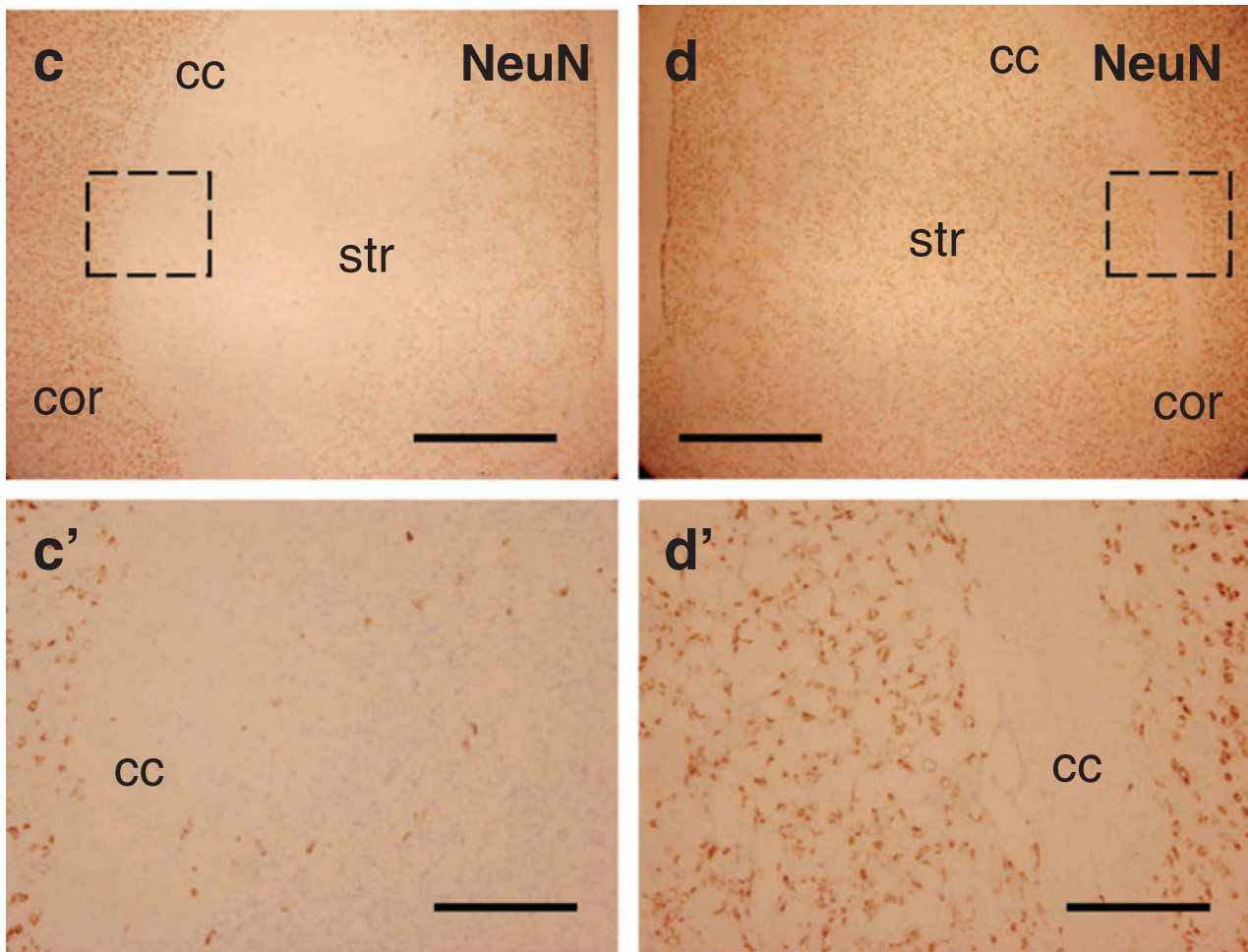


Figure 1. (Continued)

Fluorescent Microscope. The size of the ischaemic lesion was measured with a Quantimet system (Leica). For this purpose, every 5th Iba-1-stained coronary brain section (14 μ m) was captured on a computer screen. After manually outlining the clear edges of the darkly Iba-1-stained lesion, the Quantimet software calculated the size of the lesion. The total infarct volume was calculated by summation of the infarcted area of all brain sections, including the interpolated area of the four intermediate sections. For cell counting of stained brain sections captured on a computer screen, Image J software (NIH Image, Bethesda, MD, USA) was used. Data were analysed and plotted in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

Statistical analysis Statistical tests were performed in Statistica 7.0 (Statsoft, Inc., Tulsa, OK, USA). All data were rank-transformed, because the data were not normally

distributed. The course of the data was examined with an analysis of covariance. Differences between groups were analysed using a *post hoc* Turkey pair-wise comparison. Significant differences were accepted for $P < 0.05$.

Results

Ischaemic lesion after 2 weeks

Two weeks after MCAO of 1 h, brain sections showed a bean-shaped area of degenerated neurons revealed by the anti-NeuN neuronal nuclear immunostaining (Figure 1c,d). In the core of the lesion, no NeuN-positive viable neurons were detectable. In reaction to the tissue degeneration, activated Iba-1-positive microglia had invaded the entire ischaemic lesion area (Figure 1a,b). At this stage, that is, 2 weeks after MCAO, it was not possible

to discriminate anymore between the core and the penumbra of the ischaemic lesion. The ischaemic lesion was confined to the striatum, laterally and dorsally bordered by the corpus callosum and ventrally by the anterior commissure. The lesioned area in the striatum stretched along a length of approximately 4 mm, starting rostrally at Bregma 1.6 up to Bregma -2.2 [15]. The area affected by the ischaemia comprised a mean volume of 92 mm^3 (± 28). In the BMSC-treated rats too, the lesioned area was restricted to the striatum (Figure 2). Here too, the lesioned area lacked NeuN-positive viable neurons and was packed with activated Iba-1-positive microglia; no difference in microglia density could be detected between the BMSC-treated rats and the saline-treated ones. The size of the lesion in the BM-treated rats was significantly reduced to 40 mm^3 (± 18) (Figure 2c). Surprisingly, analysis of the presence of transplanted BMSCs 2 weeks after MCAO and BMSC treatment revealed only very sporadic mini-Ruby-labelled BMSCs in and around the lesion site, in the parenchyma and in blood vessels (Figure 2d). None of these labelled cells showed the expression of a marker for neural cell types. Only the spleen, but not lung or liver tissue, contained a few mini-Ruby-labelled BMSCs after 2 weeks.

Early kinetics of BM cells in ischaemic lesions

As very few of the implanted BMSCs could be found in or around the striatal ischaemic lesion at 2 weeks after implantation, we investigated the fate and kinetics of BMSCs in the first 24 h after arterial administration in correlation to the development of the ischaemic lesion in the striatum as a consequence of MCAO. To that purpose, we fixed MCAO rats at 3, 6, 12 and 24 h after BMSC administration.

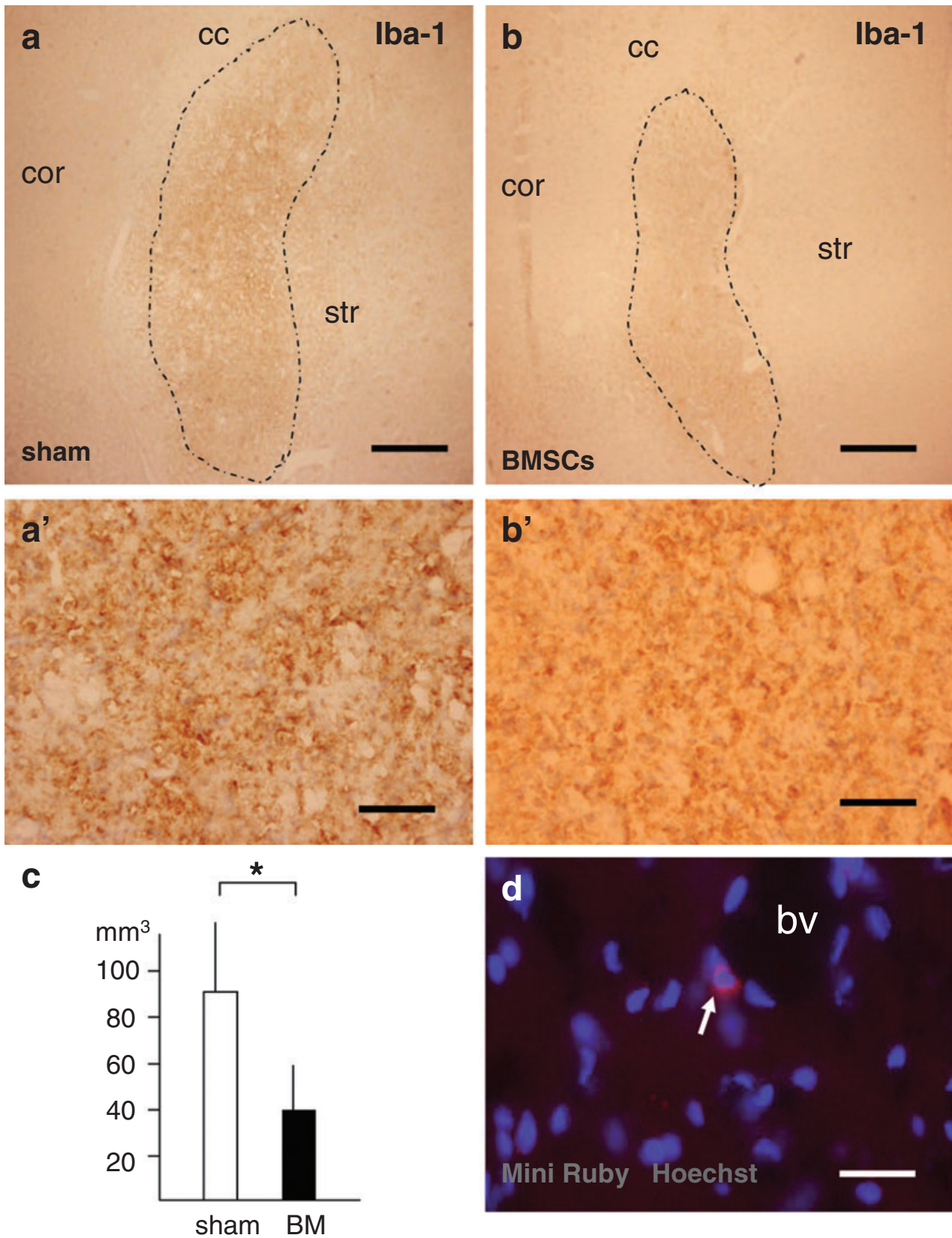
Development of striatal ischaemic lesion Analysis of sections of the striatum stained with cresyl-violet for a general overview showed that most of the tissue disruption and necrosis in the striatum developed between 12 and 24 h after BMSC administration (Figure 3). The first

apoptotic neurons inside the ischaemic lesion, as indicated by activated caspase-3 immunostaining, could be detected at 12 h (Figure 3e,f). However, the first indication for the onset of local striatal cell damage was provided by the first appearance of activated microglia in the striatum at 6 h. At 24 h, clear accumulation of activated microglia could be detected in the lesioned striatum (Figure 4). The described pattern of lesion formation in the striatum and microglia accumulation/activation was seen in BMSC-treated rats as well as in saline-treated animals.

Fate of implanted BMSCs BMSCs were administered 2 h after the onset of MCAO, that is, during the development of the ischaemic lesion. To determine the fate of the majority of the implanted BMSCs after arterial administration, we have examined filtering peripheral organs, such as lungs, liver and spleen, at the four time points: already at 3 h after BMSC administration, rhodamine-dextran-positive and CD45/CD34-positive BMSCs could be detected in follicle centres within the spleen (Figure 5). Only sparsely injected BMSCs were observed in the other tissues. The number of grafted cells that could be detected in the spleen during the time period studied, that is, up to 24 h after implantation, was counted in randomly chosen sections along the length of the spleen. At 12 h, on average 16 cells were found per spleen section, accounting in the total spleen for approximately 9.65×10^5 , that is, >95% of the number of BMSCs implanted.

Although labelled BMSCs could be detected in the brain at 3 h after BMSC administration inside blood vessels (Figure 6a,b), the first BMSCs that were observed within the striatal tissue in and around the lesion site appeared at 6 h after implantation, concomitant with the appearance of activated microglia (Figure 6c). Their number increased at 12 h after implantation. Most of the grafted BMSCs were located in and around the lesioned area in close vicinity of activated microglia. At 24 h, some of these microglia showed phagocytotic activity and contained rhodamine-dextran inclusions (Figure 6f). The number of labelled BMSCs in and around the striatum

Figure 2. Ischaemic lesion in striatum 2 weeks after middle cerebral artery occlusion (MCAO) in bone marrow stem cell (BMSC)-treated and saline-treated rats. (a,a') Iba-1 immunostaining (brown) of activated microglia reveals the size of the lesion in the left side of the striatum of saline-treated rats (magnification in a'). (b,b') Rats that received an intra-arterial injection of BMSCs 1 h after MCAO show a smaller lesion size as delineated by Iba-1 microglia (magnification in b'). (c) Measurement of the lesion sizes in saline-treated and BMSC-treated rats show a large reduction in lesion size in the BMSC-treated animals in comparison with the saline-treated rats. (d') Only sporadically could a mini-Ruby-labelled transplanted BMSC (arrow) be detected in the lesioned striatum 2 weeks after MCAO and BMSC injection (bv, blood vessel; cor, cortex; cc, corpus callosum; str, striatum). Bars in a and b, 0.5 mm; in a', b' and d, 100 μm .



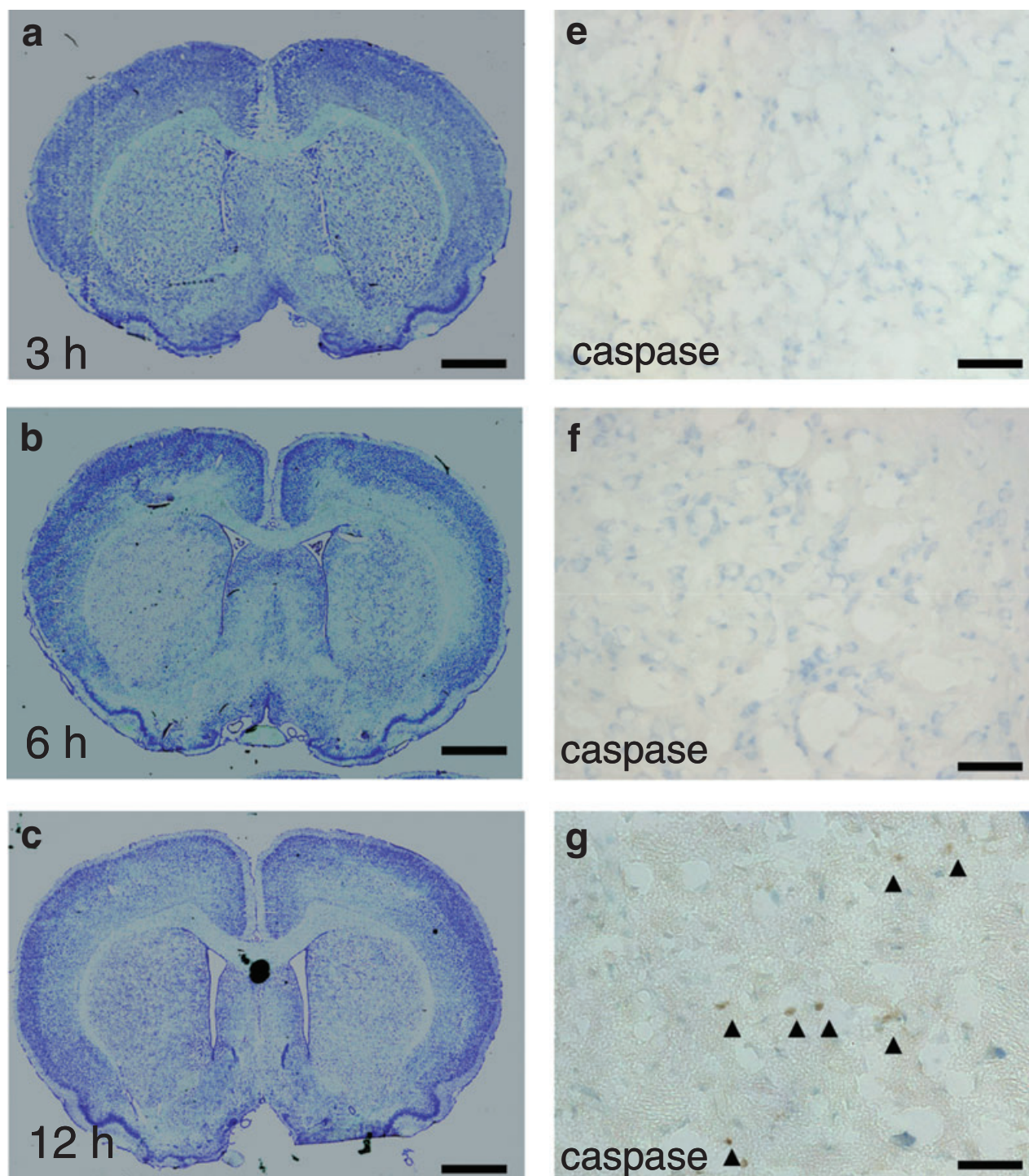


Figure 3. Overview of striatum during the first 24 h after middle cerebral artery occlusion (MCAO) and bone marrow stem cell (BMSC) implantation. (a–d) Cresyl-violet staining of striatal sections reveals the appearance of structural damage in the striatum at 12 h after MCAO and BMSC implantation; the lesioned area (arrow) is most clear in a section taken at 24 h after MCAO and BMSC injection. (e–h) Immunostaining for activated caspase-3 (brown) in sections taken from the MCAO-affected striatum shows the appearance of apoptotic cells (arrowheads) at 12 h after MCAO and BMSC injection; the number of apoptotic cells increased during the next 12 h (h, arrowheads). Bars in a–d, 1 mm; in e–h, 100 μ m.

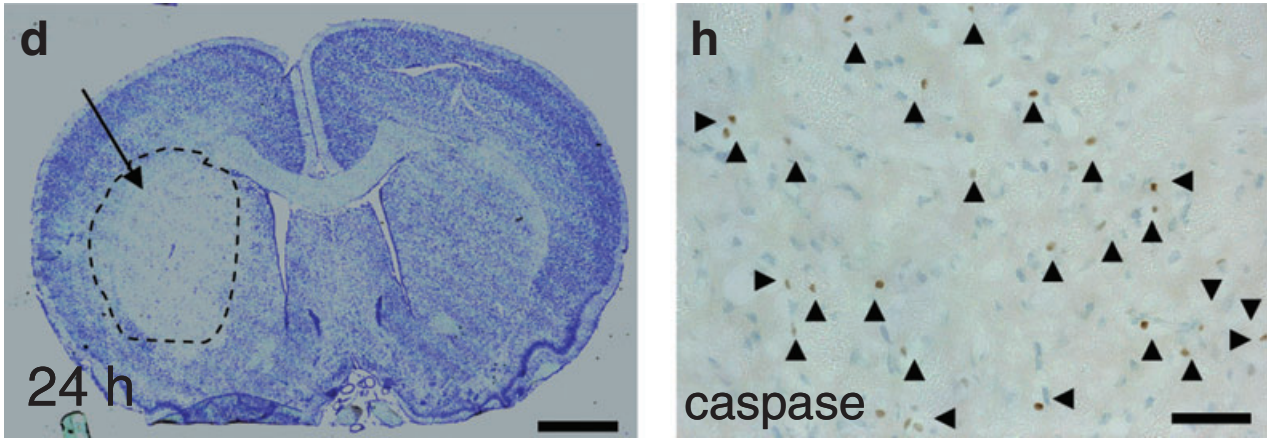


Figure 3. (Continued)

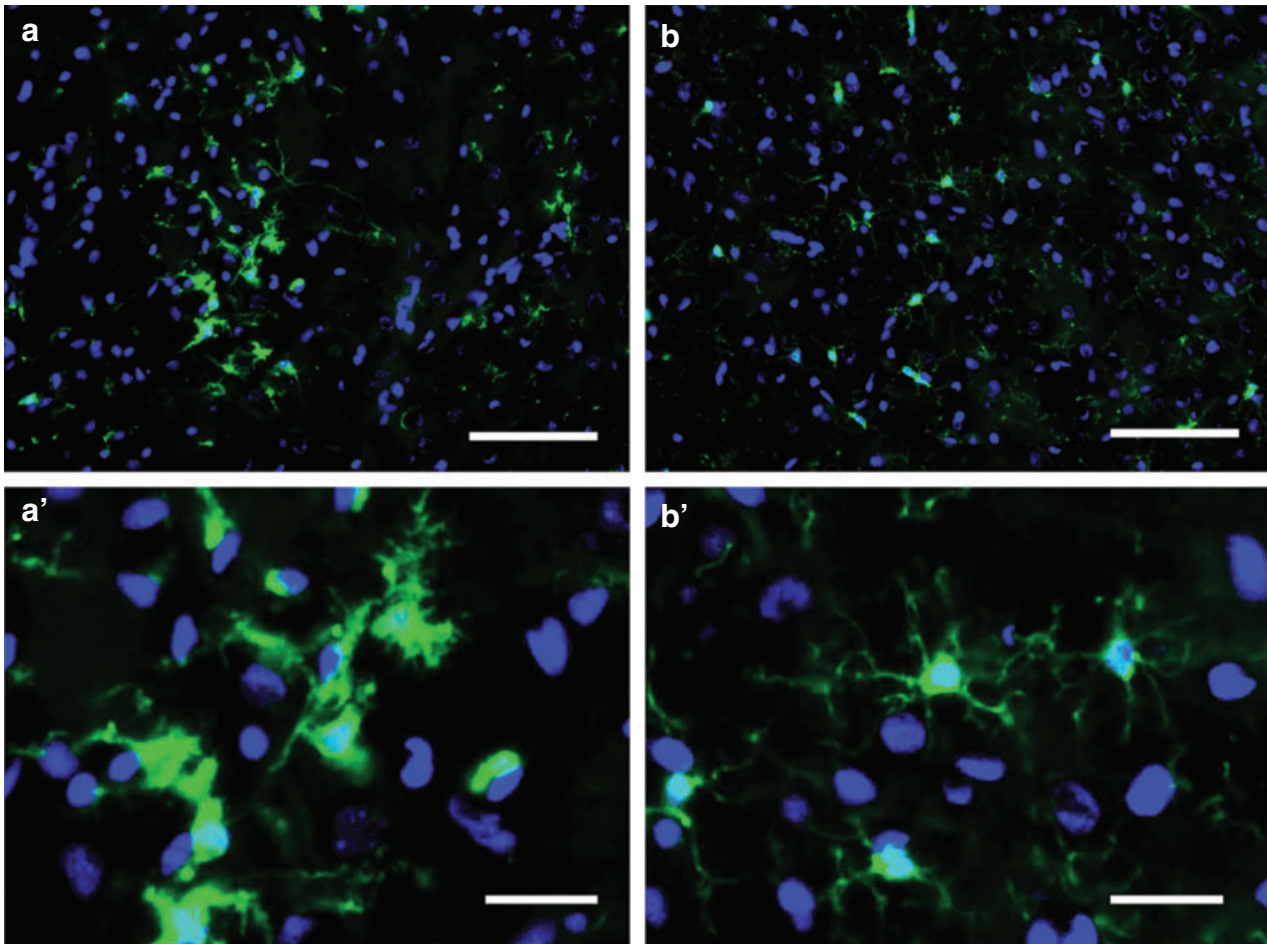


Figure 4. At 24 h after middle cerebral artery occlusion and bone marrow stem cell injection, accumulation of activated, Iba-1-positive microglia were observed at the lesioned striatum (**a**; detail in **a'**); the contralateral striatum contained an even-distributed population of quiescent microglia (**b**; detail in **b'**). Bars in **a** and **b**, 100 μ m; **a'** and **b'**, 50 μ m.

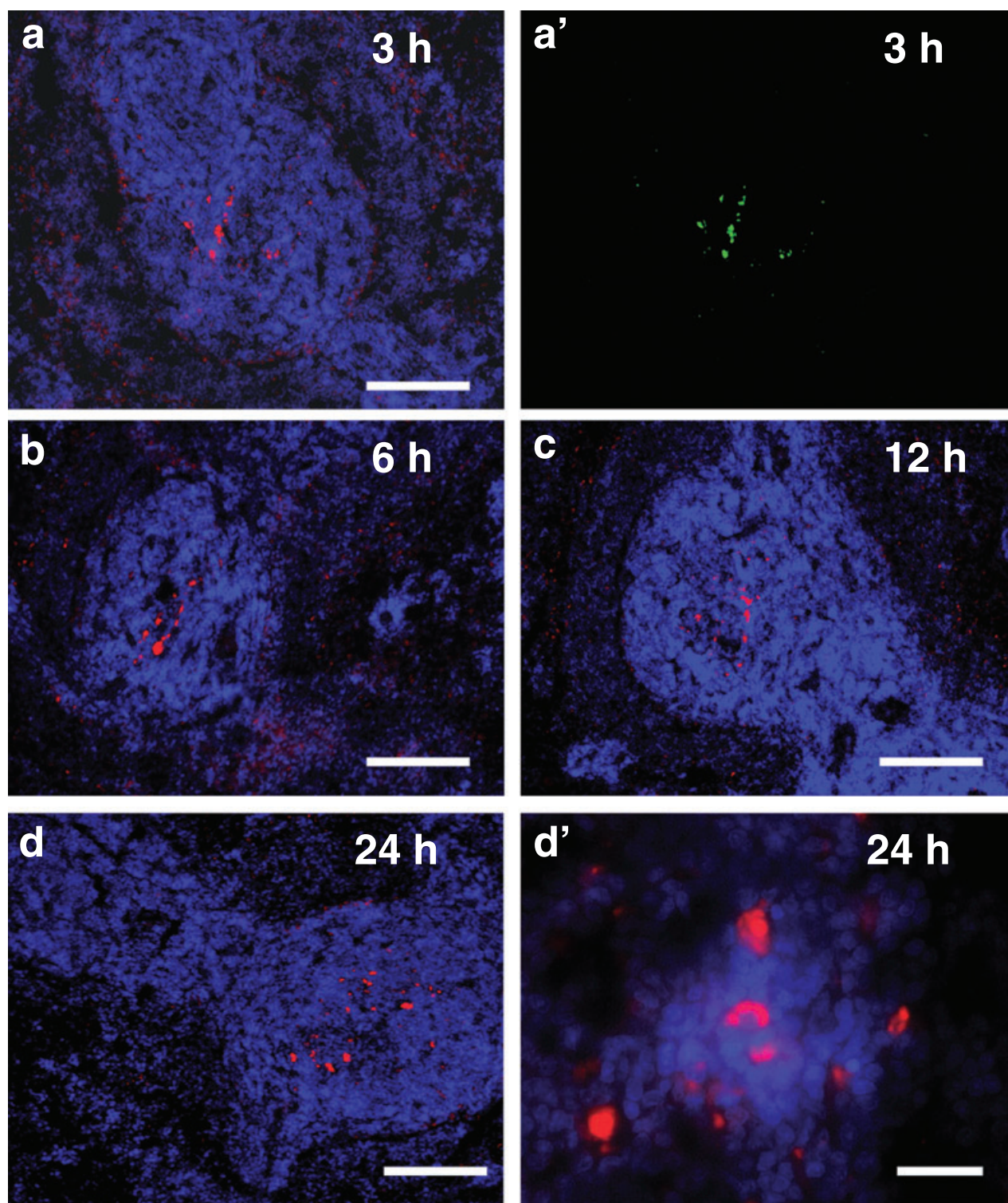


Figure 5. Grafted bone marrow stem cells (BMSCs) in spleen. (a) Mini-Ruby (red-fluorescent) and (a') CD45 (green-fluorescent) double-labelled BMSCs could be detected in follicles in the spleen already at 3 h after intra-arterial administration. (b–d) Their number gradually increased during the first 24 h after injection (d', detail of 24-h spleen follicle). At 12 h, on average 16 cells were found per spleen section, accounting in the total spleen for >95% of the implanted cells; blue, Hoechst nuclear staining. Bars a–d, 0.5 mm; d', 50 μ m.

lesion found at 24 h after implantation was slightly lower than at 12 h (Figure 6e).

Discussion

Intra-arterial injection of syngeneic BMSCs 2 h after the onset of MCAO leads to a significant reduction to approximately 50% of the ischaemic lesion. However, histological evaluation 2 weeks later could not demonstrate the presence of implanted BMSCs in or around the lesion site. Analysis of the early kinetics of BMSCs during the first 24 h after injection revealed that starting from 6 h after MCAO, concomitant with the appearance of activated microglia, a small number of BMSCs temporarily invaded the lesion site. The vast majority of implanted cells, however, appeared to be trapped in the spleen.

Our findings regarding the reduction of the lesion size are in line with the data of Iihoshi *et al.* [12]. They showed a clear correlation between lesion size and the time point of intravenous stem cell administration: whereas an acute injection 3 h after MCAO induction almost annihilated lesion formation, later administration resulted in a proportionally less reduced ischaemic lesion size [12]. We administered the BMSCs even earlier, at 2 h after ischaemia onset and, although this did lead to a significant lesion reduction, it was not as prominent as shown by Iihoshi and co-workers [12]. Apparently, earlier intravascular administration of BMSCs is suboptimal and it can be argued that at an earlier time point, that is, during a very early stage of lesion formation, signals that attract the BMSCs towards the lesion are still too low and inefficient and so most of the implanted BMSCs became trapped into the spleen. Two weeks after administration, we were unable to detect implanted BMSCs in the lesioned striatum. Obviously, we may have missed unlabelled BMSCs but, to limit this possibility, we had chosen to use a rhodamine-dextran conjugate to label our BMSCs. This type of labelling is easy, far more efficient (>98%) and less stressful for cells than most other labelling strategies. The intracellular presence of rhodamine-dextran conjugates can be detected for up to 6 months and it is presently used in combination with gadolinium as a superior novel contrast agent and fluorescent marker to track implanted stem cells both by magnetic resonance imaging and fluorescence microscopy [16,17].

The absence of implanted BMSCs 2 weeks after MCAO and intravascular injection in the striatum seems to exclude the possibility that neural cell replacement in the

striatum by BMSCs accounts for the significant ischaemic lesion size reduction observed. Apparently, the early transient presence of an, although small, number of implanted BMSC in the early phase of lesion formation caused a reduction in ischaemic lesion size. Potential players in that mechanism may be activated microglia. Reactive microgliosis after MCAO is characterized by a stereotypical graded response, including microglial activation, expansion and migration. In both saline-treated and BMSC-treated rats, we were able to detect the massive accumulation of activated microglia in the lesioned striatum 2 weeks after MCAO. Although this microglial response has been considered to contribute to neuronal damage [18–20], a growing line of evidence suggests a neuroprotective role for microglia and their neuroimmune agents in various neuropathological conditions [21,22], among which are ischaemic injury and oxygen-glucose deprivation [23–26]. Recently, Lalancette-Hebert *et al.* [27] demonstrated that ablation of proliferating microglial cells exacerbated ischaemic injury in the brain. The neuroprotective potential of the microglia appeared to be mediated by the secretion of neurotrophic and anti-apoptotic molecules, such as IGF-1 [27]. A similar neuroprotective neurogenic response of microglia mediated by IGF-1 appears to be triggered by specific cytokines [28]; some of these cytokines have been shown to be produced by BMSCs. Although the number of implanted BMSCs at the lesion site was very small and transient, they may have effectively and temporarily instructed activated microglia to a stronger neuroprotective anti-apoptotic response, resulting in a smaller ischaemic lesion. However, so far, immunohistochemical analyses (IGF-1, TGF β , BDNF, IL-4, IL-10 and IFN- γ) by us to identify a different activation response of microglia by the implanted BMSCs remained inconclusive (data not shown). Besides the microglia, implanted BMSCs themselves have been shown to secrete active agents, such as VEGF, bFGF and BDNF, which may promote angiogenesis and neurogenesis [8–10] and so contribute to lesion size reduction.

In conclusion, we have shown that the transient presence of a small number of intra-arterially administered BMSCs at an early stage of ischaemic injury can significantly reduce the ultimate lesion size.

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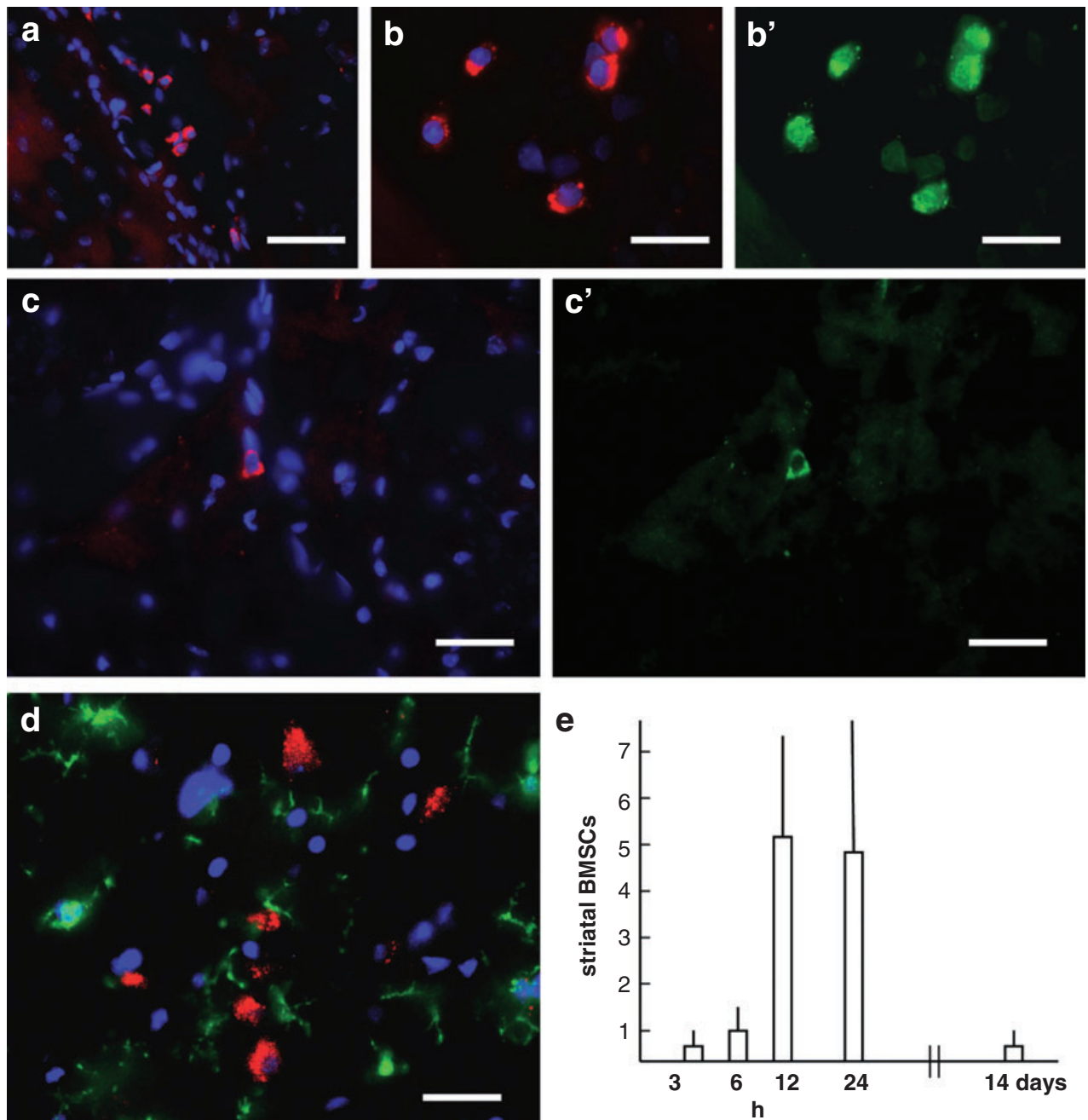


Figure 6. Grafted bone marrow stem cells (BMSCs) in the brain. (a) At 3 h after injection, rhodamine–dextran-labelled BMSCs could be detected in a few large bloodvessels; (b,b') most of these cells were co-immunostained for CD45. (c,c') At 6 h, only sporadically could a red-fluorescent bone marrow stem cell (c', CD45-positive) be detected in the lesioned striatum. At 12 (d) and 24 h (f; indicated area magnified in f'), an increase in the number of rhodamine–dextran-labelled BMSCs could be detected in the lesioned striatum. In some sections, activated Iba-1-positive (green) microglia contacted the grafted BMSCs and some of them contained rhodamine–dextran inclusions (arrows in f'). Blue, Hoechst nuclear staining; red, rhodamine–dextran; green, Iba-1. (e) Quantification of the number of grafted BMSCs present in the lesioned striatum at 3, 6, 12 and 24 h and 2 weeks after intra-arterial implantation. Bars indicate the mean number of rhodamine–dextran-labelled BMSCs (\pm standard deviation) per section of lesioned striatum, determined in three rats. Bars in a, 100 μ m; in b–f: 50 μ m; in f', 20 μ m.

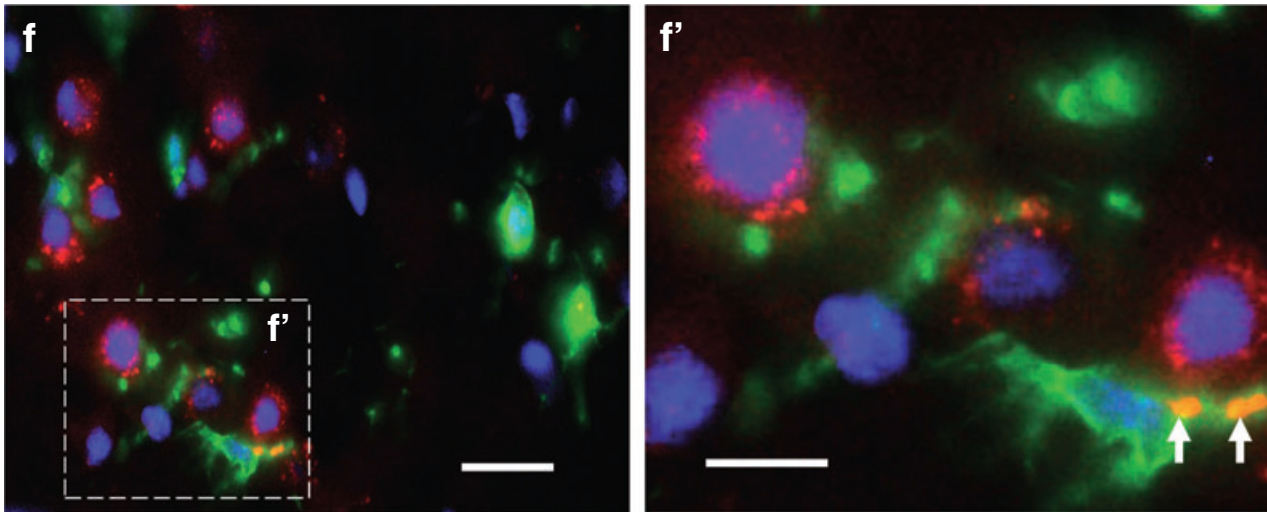


Figure 6. (Continued)

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